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(54) Title: A PEPTIDE, IMMUNOGENIC COMPOSITION AND VACCINE OR MEDICINAL PREPARATION; A METHOD OF IMMUNISING A MAMMAL AGAINST LHRH, AND A METHOD OF IMPROVING THE MEAT QUALITY OF PIGS

(57) Abstract

This invention relates to a peptide suitable for vaccination of mammals, such as pigs, against the hormone LHRH and comprising at least two LHRH sequences in tandem.

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Title: A peptide, immunogenic composition and vaccine or medicinal preparation; a method of immunising a mammal against LHRH, and a method of improving the meat quality of pigs

This invention relates to a peptide suitable for realising a vaccine effective against LHRH, the "Luteinising Hormone Releasing Hormone", also referred to as the "Gonadotrophin Releasing Hormone" (GnRH).

The invention further relates to immunogenic compositions and vaccine or medicinal preparations (vaccines and pharmaceuticals) based on such a peptide and to the use of such a vaccine or medicinal preparation in a method of immunising a mammal against LHRH and in a method of improving the meat quality of pigs.

The hormone LHRH is a small peptide being 10 amino acids in length (i.e. a decapeptide) and having an amino acid sequence according to the formula (with, as usual, the amino terminal amino acid to the left and the carboxy terminal amino acid to the right):
pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2,
in which the amino acids are designated according to the three-letter code, or according to the formula: #E H W S Y G L R P G@, in which the amino acids are designated according to the one-letter code, #E is pyroglutamic acid and G@ is glycine amide.

It is known that the LHRH, if coupled to a carrier protein, can be used to vaccinate mammals. Such a vaccination can be carried out for different reasons which are all connected with the natural function of the LHRH. The LHRH formed in the hypothalamus regulates in the hypophysis the formation of the sex hormones LH (i.e. "Luteinising Hormone") and FSH ("Follicle Stimulating Hormone"). As is known, a drastic reduction of the amounts of LH and FSH in the blood results in that in the male animal the production of androgens and sperm in the testis are inhibited and that in the female animal the formation of progestagens and oestrogens and the follicle maturation in the ovary are inhibited. Such a reduction of the amounts of androgens, progestagens and

oestrogens in the blood to a level comparable to the level obtainable by castration or ovariectomy can be realised by effective immunisation of the animal against LHRH. In veterinary medicine, 100% effective immunisation could be used for the sterilisation of, e.g., small domestic animals such as male and female cats, or for the treatment of aggressiveness in male dogs, instead of drastic surgeries such as castration and ovariectomy. Other conceivable objects of immunisation against LHRH are to prevent heat in dogs and restlessness in steer fattening. In human health care, immunisation against LHRH can be used in the treatment of prostate cancer and breast cancer and, if required, in the treatment of some forms of hypophyseal carcinoma.

Another use of a vaccine against LHRH is in the field of stock breeding, particularly the fattening of pigs for slaughter. The meat of male, sexually mature pigs ("boars") has a typical odour, the so-called "boar odour". In the sexually mature pig there are formed in the testes many steroids which are stored in the fat tissue. These steroids (C 19 Δ 16 steroids) are responsible for the unpleasant urine-like "boar odour" formed when the meat is heated (see Brooks et al, J. Anim. Sci. 62, 1279 (1986). Owing to this unpleasant odour, meat of male, sexually mature pigs is hardly, if at all, suitable for consumption. Because about 10% of the male slaughter pigs are already sexually mature before the slaughter time, this potential entails a great loss for the pig farming industry. In order to check this loss, all male pigs are castrated when they are young, without stunning. Apart from the animal unfriendly aspect of such a castration, castration also leads to growth inhibition and a final meat quality inferior to that of an intact animal (at least as long as that intact animal has not yet developed boar odour).

An animal friendly alternative, which, in addition, benefits the meat quality, consists in the immunisation of the young animal against LHRH, thereby reducing the LHRH concentration in the hypophysis of the young animal. This

reduction of the LHRH concentration in the hypophysis leads to a reduction of the concentrations of biologically active LH and FSH in the blood, which in turn results in that the development of the testes in the growing animal is prevented or delayed and that fewer steroids are formed. By effective vaccination against LHRH, it can therefore be avoided that the undesirable boar odour develops in pigs before the slaughter date.

It actually turns out in practice that in male animals the development of the testes can be delayed or stopped by vaccination with LHRH coupled to a carrier protein. The results, however, are often variable when use is made of the known vaccine preparations, such as those based on LHRH itself or on an analogue thereof, such as [D-Trp^6] LHRH (see Chaffaux et al, Recueil de Médecine Vétérinaire 161, 133-145 (1985)). For instance, there may be vaccinated animals which hardly, if at all, react to the vaccination. In the case of use in male young pigs to prevent development of boar odour, it is required for a good vaccine that in all pigs the development of the testis is delayed to the extent that in no case (up to 35 weeks after birth) boar odour develops, not even in a very large pig population. The known vaccine preparations do not meet this requirement.

This also applies to the immunogenic LHRH vaccines described in U.S. patent 4,608,251. The vaccines proposed therein are based on a nona- or decapeptide having the formula (C) K W S Y G L R P G@, or on a dimer of the decapeptide which can be formed by coupling via the amino terminal cysteines and satisfies the formula

C K W S Y G L R P G@
|
C K W S Y G L R P G@

This dimer does not seem to be more efficient than the monomeric peptides.

The international patent application WO 88/05308 proposes LHRH vaccines based on partial peptides of the LHRH having a length of 5, 6 or 7 amino acids, particularly those peptides

which comprise either the amino terminal pGlu or the carboxy terminal Gly-NH_2. Examples of such partial peptides are (given in the one-letter code) #E H W S Y, #E H W S Y G, #E H W S Y G L, H W S Y G L R, W S Y G L R, S Y G L R P G@, and Y G L R P G@. However, the vaccines based on these partial peptides also show the drawback mentioned before.

Surprisingly, it has now been found that a better and particularly more reliable vaccine is obtained if it is based on a peptide having an LHRH tandem structure, i.e. a peptide comprising at least 2 LHRH sequences arranged one behind the other.

Consequently, the invention first resides in a peptide which is characterised in that it comprises at least 2 LHRH sequences in tandem.

According to the invention there is preferred a peptide which is characterised in that it comprises at least 2 LHRH sequences in tandem according to the general formula (with the amino terminal amino acid to the left and the carboxy terminal amino acid to the right)

Z^1-Glx-His-Trp^1-Ser-Tyr-Gly-Leu-Arg-Pro[-Gly-X-Gln-His-Trp^2-Ser-Tyr-Gly-Leu-Arg-Pro]$_n$-Gly-Z^2,

in which amino acids are designated according to the three-letter code, Trp^1 and Trp^2 are tryptophan (Trp) or formylated tryptophan ($N_{(indole)}$-formyl-tryptophan),

n is a number having a value of at least 1,

X is either a direct bond or a spacer group between the amino acids Gly and Gln,

Z^1-Glx is either pGlu (pyroglutamic acid) or Gln having attached thereto a tail comprising one or more additional amino acids, and

Gly-Z^2 is either Gly-NH_2 or Gly having attached thereto a tail comprising one or more additional amino acids.

In this general formula, X may be a direct bond between the amino acids glycine and glutamine, i.e. these amino acids are interconnected directly without an intermediate link (via the normal peptide bond). Although this is preferred indeed,

the invention also comprises peptides in which the LHRH sequences are interconnected via spacers. The nature of the spacer group may greatly vary from one or more amino acids to a shorter or longer hydrocarbon chain and other compound groups or molecules.

In the above general formula, Z^1-Glx preferably stands for pGlu (pyroglutamic acid), but can also stand for Gln having attached thereto a tail comprising one or more additional amino acids, e.g., to be used for coupling of the peptide to a carrier protein.

In the above general formula, Gly-Z^2 stands for, e.g., Gly-NH_2, or Gly having attached thereto a tail comprising one or more additional amino acids, e.g., to be used for coupling of the peptide to a carrier protein. Preferably, Gly-Z^2 stands for Gly-Cys-NH_2, the C terminal cysteine being added in connection with a possible coupling of the peptide to a carrier protein.

More in particular, there is preferred according to the invention a peptide which is characterised in that it comprises at least 2 LHRH sequences in tandem according to the general formula (with the amino terminal amino acid to the left and the carboxyterminal amino acid to the right)
pGlu-His-Trp^1-Ser-Tyr-Gly-Leu-Arg-Pro[-Gly-Gln-His-Trp^2-Ser-Tyr-Gly-Leu-Arg-Pro]$_n$-Gly-Cys-NH_2,
in which amino acids are indicated according to the three-letter code, Trp^1 and Trp^2 are either Trp or N-formyl-Trp, and n is a number having a value of at least 1.

A concrete example of such a preferred peptide is the peptide which comprises 2 LHRH sequences in tandem according to the formula (with the amino terminal amino acid to the left and the carboxy terminal amino acid to the right)
pGlu-His-Trp^1-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Gln-His-Trp^2-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Cys-NH_2,
in which amino acids are designated according to the three-letter code, and Trp^1 and Trp^2 are either Trp or N-formyl-Trp.

The invention further resides in a composition which is characterised in that it comprises a peptide according to the invention brought into an immunogenic form. As a skilled worker knows, there are different methods of bringing a substance which by itself is not immunogenic, into an immunogenic form. A first possibility is to couple a peptide according to the invention to a suitable carrier protein. For a chemical coupling the C or the N terminus can be suitably used. Those skilled in the art perfectly know what coupling methods and what carrier proteins are eligible. See in this connection, e.g., the above cited US-A-4,608,251 and WO 88/05308. According to the invention there is preferred a composition which is characterised in that it comprises an immunogenic conjugate of a protein and a peptide according to the invention. Another possibility is to convert the peptide by crosslinking into some larger complex, or to express the peptide by means of recombinant DNA manipulations as part of a (larger) protein. Ther invention therefore also resides in a composition comprising an immunogenic complex or an immunogenic recombinant protein to which a peptide according to the invention belongs.

Of course, the invention also resides in a vaccine or medicinal preparation which is characterised in that it comprises such a composition according to the invention in combination with at least one immunoadjuvant. Suitable immunoadjuvants are known to those skilled in the art and comprise, e.g., CFA (Complete Freund's Adjuvants) and IFA (Incomplete Freund's Adjuvants).

The invention further provides a method of immunising a mammal against LHRH, which method is characterised in that said mammal is vaccinated with such a vaccine or medicinal preparation according to the invention. Reasons for such a vaccination have already been indicated above, such as the use in human medicine for the treatment of prostate cancer and breast cancer and of some forms of hypophyseal carcinoma, various uses in veterinary medicine and various uses in stock-

breeding. A special use is within the scope of a method of improving the meat quality of pigs, which is characterised in that said pigs are vaccinated with such a vaccine preparation according to the invention.

The invention will be explained in more detail with reference to the following practical example.

Example

Three groups of male young pigs (5 animals per group) were vaccinated with an LHRH vaccine after birth (day 0). The vaccines consisted of a peptide coupled to the carrier protein KLH (Keyhole Limpet Hemocyanine) via a C terminal cysteine. This coupling was effected by means of MBS (the compound m-maleimidobenzoyl-N-hydroxy succinimide ester, see Geysen et al, PNAS 81, 3998-4002 (1984)).

The peptides used were:

group I: LHRH

formula #E H W S Y G L R P G C@

group II: [D-Trp^6]LHRH

formula #E H W S Y [D-Trp] L R P G C@

group III: tandem LHRH

formula #E H W S Y G L R P G Q H W S Y G L R P G C@

The peptides were emulsified after coupling to KLH (1 mg peptide to 1 mg KLH) in 1 ml CFA (1 ml peptide-KLH solution in 1 ml CFA). The emulsion was injected intramuscularly on day 0, and also 8 weeks later. During the second vaccination IFA was used.

A control group (group IV) was only vaccinated with the carrier protein (KLH) and adjuvant.

On day 0, and 4, 8 and 12 weeks later, the size of the testes was measured. On 17-18 weeks the animals were slaughtered. On 12 weeks after the first vaccination the testerone content was measured in the serum of the pigs. On 131 days after the first vaccination the testis and epididymis weights and the seminal vesicle and bulbourethral weights of the pigs were determined.

The results of the four groups were as follows. After a vaccination with LHRH (group I) external measurement showed that three out of five animals had smaller testes than the controls (group IV). Of the three animals having smaller testes, two had testes that had decreased in size to the extent that they could no longer be measured externally.

Of the animals vaccinated with [D-Trp^6]LHRH (group II), the testes of two out of five animals were clearly smaller than in the control animals, and in one animal the testes were no longer measurable.

Of the five animals vaccinated with tandem LHRH (group III), all the testes were smaller than in the control animals, and in four animals the testes were no longer measurable.

The mean testosterone contents in the serum were respectively:

2.47 (1.21) pmol/ml (control group, 5 pigs)
1.89 (2.24) pmol/ml (group I, 5 pigs)
2.72 (2.23) pmol/ml (group II, 5 pigs)
0.51 (0.08) pmol/ml (group III, 5 pigs).

The mean testis and epididymis weights were respectively:

285 (50) g/55 (11) g (control group, 5 pigs)
110 (123) g/21 (17) g (group I, 5 pigs)
221 (88) g/42 (12) g (group II, 5 pigs)
25 (11) g/11 (2) g (group III, 4 pigs).

The mean seminal vesicle and bulbourethral weights were respectively:

135 (44) g/119 (24) g (control group, 5 pigs)
40 (80) g/37 (48) g (group I, 5 pigs)
137 (71) g/135 (52) g (group II, 4 pigs)
5 (3) g/15 (5) g (group III, 4 pigs).

The bracketed values are the standard deviations.

Since delayed growth or even regression of the testis can be directly correlated with the decline of the androgen-producing capacity of the pig and consequently with the development of the undesirable boar odour, the contemplated complete protection is realised in group III treated with a

tandem LHRH vaccine according to the invention, while only a partial protection was observed in groups I and II taken for comparative purposes.

CLAIMS

1. A peptide, characterised in that it comprises at least 2 LHRH sequences in tandem.

2. A peptide, characterised in that it comprises at least 2 LHRH sequences in tandem according to the general formula (with the amino terminal amino acid to the left and the carboxy terminal amino acid to the right)
Z^1-Glx-His-Trp^1-Ser-Tyr-Gly-Leu-Arg-Pro[-Gly-X-Gln-His-Trp^2-Ser-Tyr-Gly-Leu-Arg-Pro]$_n$-Gly-Z^2,
in which amino acids are designated according to the three-letter code, Trp^1 and Trp^2 are either Trp or N-formyl-Trp, n is a number having a value of at least 1, X is either a direct bond or a spacer group between the amino acids Gly and Gln, Z^1-Glx is either pGlu (pyroglutamic acid) or Gln having attached thereto a tail comprising one or more additional amino acids, and Gly-Z^2 is either Gly-NH_2 or Gly having attached thereto a tail comprising one or more additional amino acids.

3. A peptide, characterised in that it comprises at least 2 LHRH sequences in tandem according to the general formula (with the amino terminal amino acid to the left and the carboxy terminal amino acid to the right)
pGlu-His-Trp^1-Ser-Tyr-Gly-Leu-Arg-Pro[-Gly-Gln-His-Trp^2-Ser-Tyr-Gly-Leu-Arg-Pro]$_n$-Gly-Cys-NH_2,
in which amino acids are designated according to the three-letter code, Trp^1 and Trp^2 are either Trp or N-formyl-Trp, and n is a number having a value of at least 1.

4. A peptide, characterised in that it comprises 2 LHRH sequences in tandem according to the formula (with the amino terminal amino acid to the left and the carboxy terminal amino acid to the right)
pGlu-His-Trp^1-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Gln-His-Trp^2-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Cys-NH_2,

in which amino acids are designated according to the three-letter code, and Trp^1 and Trp^2 are either Trp or N-formyl-Trp.

5. A composition, characterised in that it comprises a peptide according to any of claims 1-4 brought into an immunogenic form.

6. A composition, characterised in that it comprises an immunogenic conjugate of a protein and a peptide according to any of claims 1-4.

7. A composition, characterised in that it comprises an immunogenic complex to which a peptide according to any of claims 1-4 belongs.

8. A composition, characterised in that it comprises an immunogenic recombinant protein to which a peptide according to any of claims 1-4 belongs.

9. A vaccine or medicinal preparation, characterised in that it comprises a composition according to any of claims 5-8 in combination with at least one immunoadjuvant.

10. A method of immunising a mammal against LHRH, characterised in that said mammal is vaccinated with a vaccine preparation according to claim 9.

11. A method of improving the meat quality of pigs, characterised in that said pigs are vaccinated with a vaccine preparation according to claim 9.

INTERNATIONAL SEARCH REPORT

International Application No PCT/NL 90/00037

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 07 K 7/20, C 07 K 7/08, A 61 K 39/385, A 61 K 37/38

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	A 61 K, C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	WO, A, 88/05308 (COMMONWEALTH SCIENTIFIC AND IND. RESEARCH ORGANISATION) 28 July 1988 --	
A	EP, A, 0181236 (PITMAN-MOORE INC.) 14 May 1986 --	
A	The Journal of Biological Chemistry, volume 263, no. 4, 5 February 1988, The American Society for Biochemistry and Molecular Biology, Inc., (Baltimore, MD, US), D.N. Posnett et al.: "A novel method for producing anti-peptide antibodies. Production of site-specific antibodies to the T cell antigen receptor β-chain", pages 1719-1725 --------	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
4th July 1990	07 AUG 1990
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Mme N. KUIPER

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 10, 11 because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv): methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods

2. [ ] Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- [ ] The additional search fees were accompanied by applicant's protest.
- [ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. NL 9000037 SA 35710

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 30/07/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8805308	28-07-88	AU-A- 1101788	10-08-88
		ZA-A- 8800149	28-06-88
EP-A- 0181236	14-05-86	US-A- 4608251	26-08-86
		AU-B- 587825	31-08-89
		AU-A- 5717886	12-11-87